ZOOLOGICAL SCIENCE 11: 693–700 (1994)

Changes at the Egg Surface during the First Maturation Division in the Spider *Achaearanea japonica* (Bös. *et* Str.)

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ABSTRACT—Changes at the egg surface during the first maturation division were examined in the spider *Achaearanea japonica* under the light and electron microscope. Newly laid eggs had already accepted a sperm nucleus. The nuclear division of the primary oocyte had occurred parallel to the egg surface and then the meiotic spindle developed perpendicular to the egg surface to complete the division. The cell membrane began to invaginate from the egg surface immediately after oviposition. Then the lumen of the invagination became wider and the large yolk granules became organized into an outer columnar layer and an inner spherical mass. The outer layer of the "vitelline membrane" appeared to be formed by the contents of periplasmic granules, which were newly detected in the present study. These granules discharged their contents by exocytosis. The formation of the outer layer after entrance of the sperm nucleus suggests that the "vitelline membrane" of the spider egg is equivalent to a fertilization membrane and is not a true vitelline membrane.

INTRODUCTION

The eggs of spiders are generally considered to be surrounded by two egg membranes: an outer membrane, the chorion; and an inner membrane, the so-called "vitelline membrane". According to Kondo [5], in lycosid spiders, a single membrane corresponding to the outer layer of the "vitelline membrane" is located between the chorion and the cell membrane 30 min after oviposition. The "vitelline membrane" then becomes distinguishable under the light microscope after formation of the main layer has started. Kondo suggested that the "vitelline membrane" bore some resemblance to the fertilization membrane. Suzuki and Kondo [13] described how, in the theridiid spider *Achaearanea japonica*, the main layer was formed under the outer layer by the matrix of vesicles that had been discharged by exocytosis. In the present study, we examined the changes that occur at the egg surface in newly laid eggs during the first maturation division in *A. japonica*. We discuss the possibility that the "vitelline membrane" could be referred to as the fertilization membrane and we identify the origin of the outer layer of the fertilization membrane.

MATERIALS AND METHODS

Mature female specimens of *Achaearanea japonica* (Bös. *et* Str.) lay eggs in middle and late summer, and about 100 eggs are released at each oviposition. The eggs are spherical and 0.5 mm in diameter. Eggs laid naturally on the campus of Toho University were used for the present study.

The eggs were covered with oviposition fluid immediately after oviposition. Before this fluid hardened, the eggs were ellipsoidal. It was difficult to isolate each egg without injury. Fixation of an entire mass was more effective at the earlier stages and, therefore, the eggs were immersed in fixative at the site of their collection.

Each egg mass was harvested 10 min after oviposition and was carried to the laboratory. The oviposition fluid usually took at least 20 min to dry up and then the eggs ceased to stick together. The physical properties of the egg membrane, such as its elasticity and permeability to fixatives, changed during the evaporation of the oviposition fluid. For example, an egg covered with the fluid was elastic and fragile while a dry egg was less elastic and firm. In the latter case, fixation after puncturing of the egg with a tungsten needle in the fixative was more effective than fixation without puncturing.

For light microscopy, eggs were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) that contained 0.2 M sucrose. Samples dehydrated in a graded alcohol series were embedded in methacrylate resin (Technovit 7100; Kulzer, Wehrheim, Germany). The resin-embedded specimens were sectioned at 1–5 μm with a glass knife on an ultramicrotome (type 4800; LKB-Produkter, Stockholm, Sweden). The sections were stained with Mayer's acid-haemalum and eosin.

For fine-structural observations, the eggs were prefixed at room temperature for 3 hr in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) that contained 0.2 M sucrose. During fixation, the eggs were cut in half with a tungsten needle. After rinsing for more than one hour with the same buffer plus 0.2 M sucrose, the samples were postfixed at room temperature for one hour in 2% osmic acid in 0.1 M phosphate buffer (pH 7.4) without sucrose. After rinsing with the same buffer without sucrose, the samples were dehydrated in a graded alcohol series, transferred to propylene oxide and embedded in epoxy resin (Quetol 812; Nisshin EM, Tokyo). Ultrathin sections were cut with a diamond knife on the ultramicrotome, stained with uranyl acetate and lead citrate and examined under an electron microscope (JEM-1210; JEOL, Tokyo). Thick sections were prepared simultaneously and these sections were stained with toluidine blue for light microscopy.

Accepted August 24, 1994
Received June 3, 1994
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RESULTS

Light microscopy

By the time oviposition occurred, the egg had already accepted a sperm nucleus which was visible at a depth of about 50 μm from the egg surface (Fig. 1). The cytoplasm was distributed at the surface of each egg as a layer of periplasm of 10–20 μm in thickness (Fig. 1). The egg contained many large yolk granules of 10–40 μm in diameter and these granules were deeply stained by eosin and by toluidine blue.

Evidence for the first maturation division of the oocyte, which was in the telophase, was visible in the periplasm (Fig. 2). The meiotic spindle was oriented parallel to the egg surface. The spindle curved toward the center of the egg in its equatorial region, resembling a cup in terms of shape.

The egg membrane appeared to consist only of a chorion with a rough external surface and a smooth internal surface (see Fig. 5).

Ten minutes after oviposition, the meiotic spindle of the first maturation division was oriented obliquely with respect to the surface in some eggs (Fig. 3), while in other eggs two sets of daughter chromosomes were oriented perpendicularly to the surface of the egg (Fig. 4a, b). In the latter case, the meiotic spindle was not clearly discernible and a deeply located set of chromosomes was visible about 40 μm from the surface of the egg.

Twenty minutes after oviposition, the large yolk granules consisted of two layers: an outer layer of radial columns and an inner layer that was a spherical mass (Fig. 5). Two chromosome plates were observed, situated close to each other (Fig. 5). One of them, located at the surface of the spherical mass of the large yolk granules, was that of the secondary oocyte and the other, located in the periplasm or in the cytoplasm accompanied by radial columns of large yolk granules, was that of the first polar body. Protrusion of the cytoplasm of the first polar body from the periplasm was not observed. The yolk mass resembled an aggregation of irregular polygons in live eggs at this stage (Fig. 6).

Electron microscopy

At the time oviposition occurred, many microvilli were observed on the surface of the egg (Fig. 7). The main components of the cytoplasm were fatty granules and vesicles. The fatty granules, 1–2.5 μm in diameter, had a moderately electron-dense matrix and their limiting membranes were often obscure. Vesicles were 1–6 μm in diameter and contained a slightly electron-opaque matrix. Some small yolk granules of less than 5 μm in diameter were observed. Both large and small yolk granules had smooth surfaces and their limiting membranes were not visible for the most part. The yolk granules were very electron-dense. Mitochondria were either oval or rod-shaped and had a very electron-dense matrix. Granules that could be divided into the following two types were detected for the first time in the present study and they were designated "periplasmic granules". Periplasmic granules of the first type were ellipsoidal with a long axis of 0.3–0.5 μm, and each contained membrane-like material (Fig. 8). Periplasmic granules of the second type were spherical, 0.2–0.4 μm in diameter, and contained a very electron-dense matrix (Fig. 8). Periplasmic granules that appeared to be intermediate between the two types were also observed (Fig. 9). The glycogen granules were very electron-dense and clusters of glycogen granules were observed near the large yolk granules.

Electron micrographs of maturation divisions were unavailable because of our failure to stain the thick, epoxy resin-embedded sections.

A structure corresponding to the "vitelline membrane", which was usually found at later stages of development, was not observed at the surface of the egg. The chorion was composed of a weakly electron-dense basal layer of 0.25 μm in thickness and electron-dense spherules of 0.5–0.8 μm in diameter (Fig. 7). The spherules were attached to the outer surface of the basal layer.

Within two minutes after oviposition, microvilli became less prominent and the periplasmic granules migrated to the outermost region of the periplasm (Fig. 10).

Three minutes after oviposition, the microvilli had disappeared entirely. The membrane-like material and the very electron-dense matrix of periplasmic granules were discharged at the egg surface by exocytosis, and the egg surface was enveloped discontinuously by a thin membrane or mucous material (Figs. 11 and 12). The cell membrane had invaginated sporadically at intervals of 25–50 μm toward the center of the egg (Fig. 13), and the tips of invaginations extended to a depth of about 25 μm from the egg's surface. Vesicles were often arranged in sequence beyond the tips of invaginations (Fig. 14).

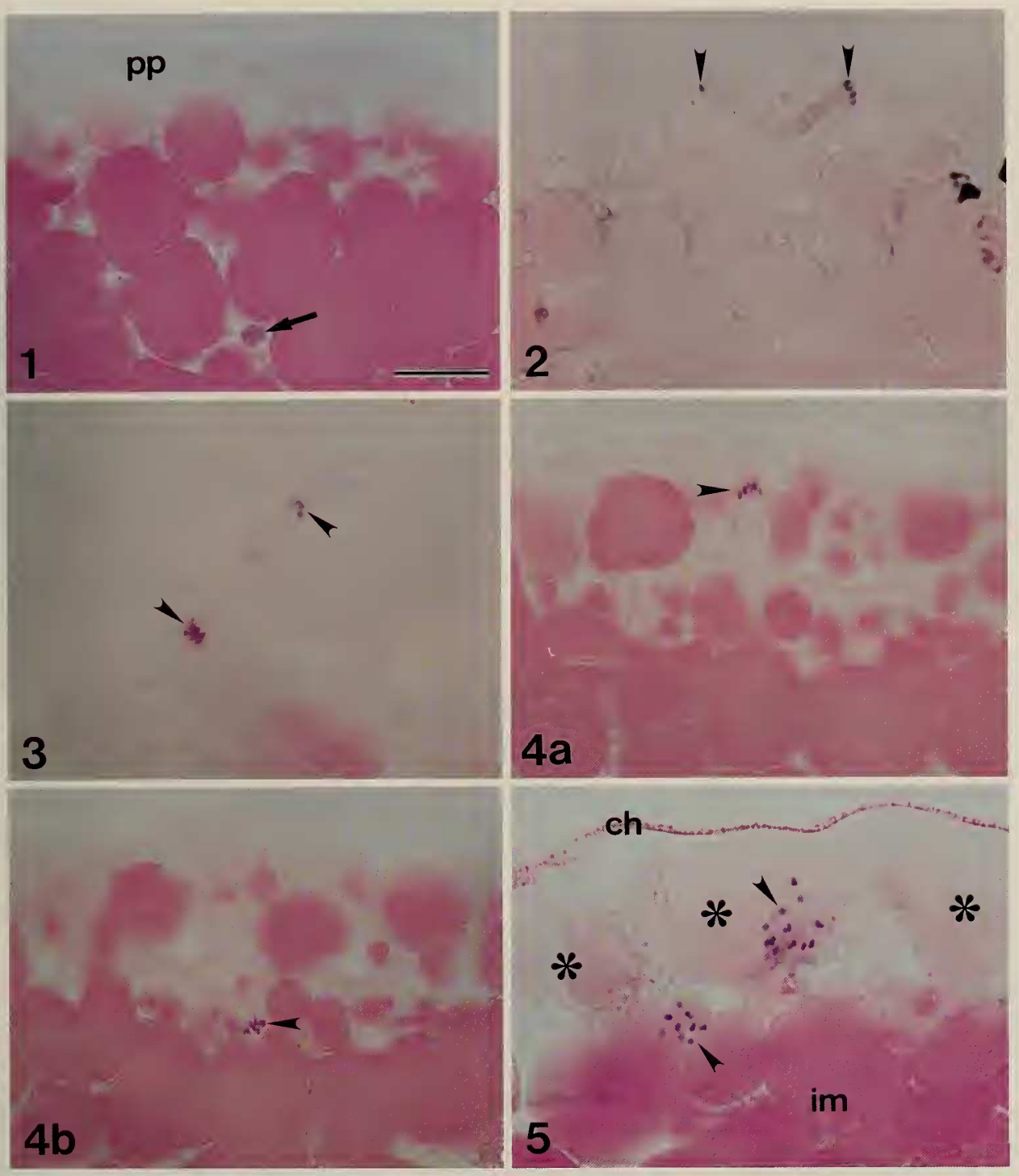
Fifteen minutes after oviposition, the surface of the egg was completely enveloped by a thin membrane and an underlying layer of mucous material (Fig. 15). The chorion was not in contact with this newly formed envelope. Exocytosis of periplasmic granules was still observed at this time.

Twenty minutes after oviposition, the cell membrane had invaginated to a distance of 25–30 μm from the surface of the egg. The lumen of each invagination became wider from a depth of about 5 μm to the tip, and large yolk granules were arranged into an outer layer of radial columns and an inner spherical mass (Fig. 16). At this time, a membrane of about 30 nm in thickness was located between the chorion and the cell membrane (Fig. 17). The discontinuity of the membrane with the chorion and the cell membrane indicated that it was the outer layer of a fertilization membrane. Microvilli were again observed but the number of periplasmic granules had decreased markedly.

DISCUSSION

The chorion

The egg membranes of spiders are considered to consist of a chorion and a "vitelline membrane". The chorion of



FIGS. 1–5. Surfaces of newly laid eggs during the first maturation division (Technovit-embedded sections). Scale bar=20 μm. arrowheads, chromosomes. Fig. 1. Just at the time of oviposition. The egg includes many large yolk granules and has accepted a sperm nucleus (arrow). The cytoplasm is distributed at the egg surface as periplasm (pp). Fig. 2. Just at the time of oviposition. The nuclear division of the primary oocyte at telophase is visible in the periplasm. The division occurs parallel to the egg surface. The spindle becomes cup-shaped. Fig. 3. Ten minutes after oviposition. The meiotic spindle lies obliquely with respect to the egg surface. Figs. 4a and 4b. Ten minutes after oviposition. Two sections from a serially sectioned egg. Two sets of daughter chromosomes are located perpendicular to the egg surface. The chromosomes in Fig. 4a will be those of the first polar body, and those in Fig. 4b will be those of the secondary oocyte. Fig. 5. Twenty minutes after oviposition. The large yolk granules are arranged as an outer layer of radial columns (asterisks) and an inner spherical mass (im). Two chromosome plates are visible. One of them, located at the surface of the spherical mass of the large yolk granules, is that of the secondary oocyte and the other, located in the periplasm and in the cytoplasm accompanied by a radial column of the large yolk granules, is that of the first polar body. ch, chorion.

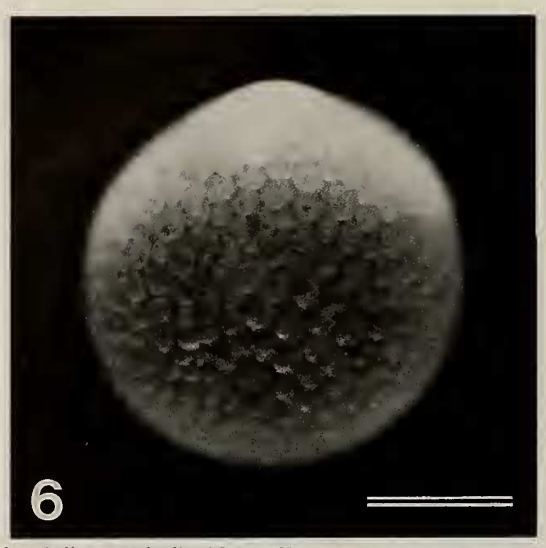


Fig. 6. A live egg in liquid paraffin, twenty minutes after oviposition. The yolk mass appears to be divided into irregular polygons. Scale bar=0.2 mm.

Achaearanea japonica consists of a basal layer and spherules. The fine structure of the chorion is similar to that of eggs of lycosid spiders [5]. No such spherules have been described in the ovarian eggs of *Heptathela kimurai* [10], *Plexippus paykulli* [11] and *Cupiennius salei* [12]. Kondo and Chaki [6] reported mature eggs that were enclosed by spherules in the ovarian cavity of *Nephila clavata*. In spiders, the spherules should attach to the basal layer of the chorion when eggs are released into the ovarian cavity.

Lambert [7] reported that, in *Epeira cinerea*, the spherules floated away freely when living eggs were immersed in alcohol and he concluded that they were not structurally a part of the chorion. This phenomenon was not observed in *A. japonica*. When live eggs of *A. japonica* were shaken in a solution of sodium hypochlorite, the spherules easily became detached from the basal layer and each egg membrane became transparent. Moreover, many craters were visible on the surface of the basal layer under the scanning electron microscope (Suzuki and Kondo, unpublished data). Each crater probably corresponded to a site at which a spherule had been attached, and attached spherules would make the egg appear opaque. Since the egg membrane of *Heptathela kimurai* is transparent [14], it would be of interest to determine whether spherules are present in the chorion of this primitive spider.

The outer layer of the fertilization membrane

It has been generally accepted that the "vitelline mem-

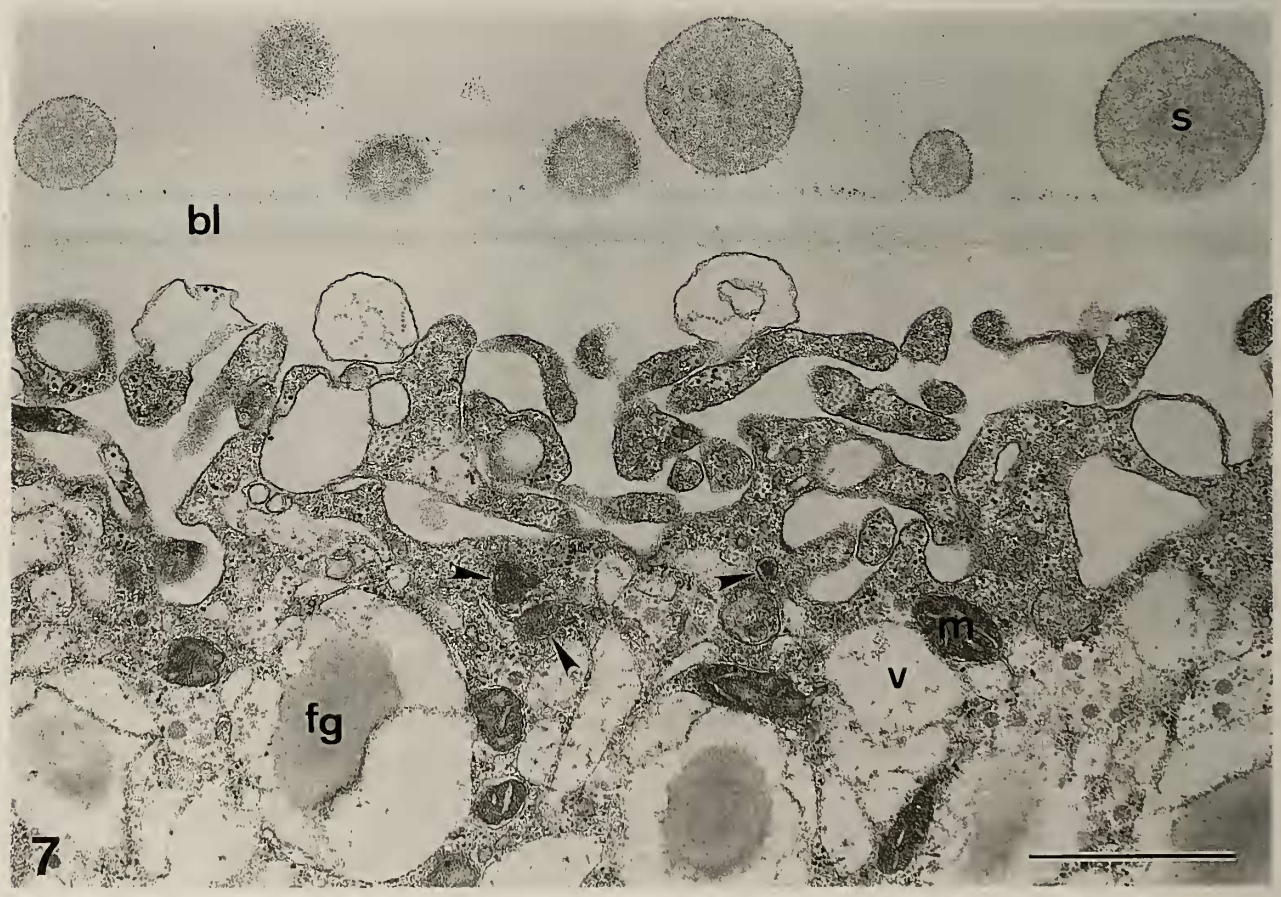
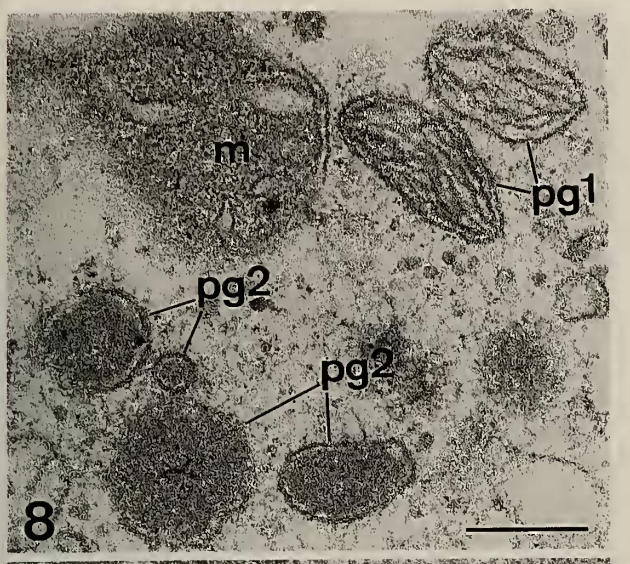


Fig. 7. The egg surface just at the time of oviposition. The chorion is composed of a basal layer (bl) and spherules (s). The cell membrane extrudes many microvilli. No "vitelline membrane" is visible under the chorion. Scale bar=1 μm. arrowheads, periplasmic granules; fg, fatty granule; m, mitochondrion; v, vesicle.



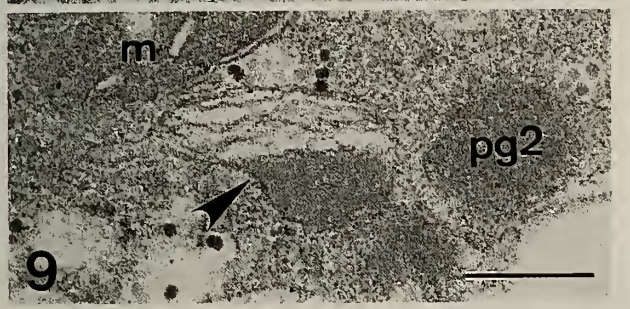


FIG. 8. Electron micrograph showing periplasmic granules. Periplasmic granules of the first type (pg1; see text) are ellipsoidal and contain membrane-like material. Granules of the second type (pg2; see text) are spherical and contain a very electron-dense matrix. Scale bar=0.2 μm. m, mitochondrion.

FIG. 9. Periplasmic granule of the transitional type (arrowhead; see text), which is intermediate between granule of the first type and that of the second type. Scale bar=0.2 μm. m, mitochondrion; pg2, periplasmic granule of the second type.

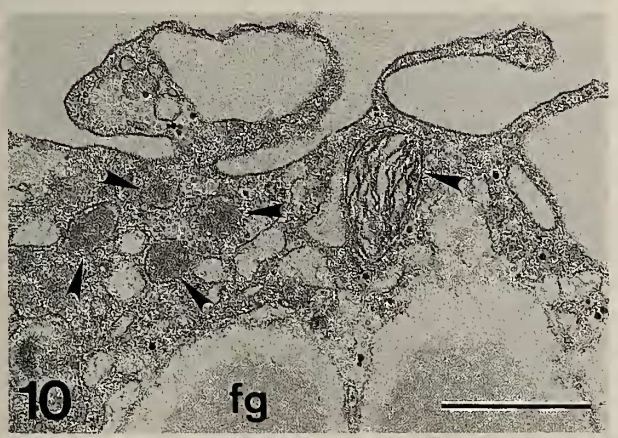
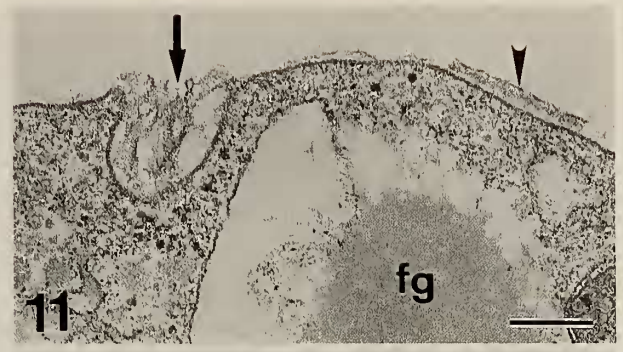


FIG. 10. The surface of the egg, two minutes after oviposition. Microvilli are faintly visible (compare with Fig. 7). Periplasmic granules (arrowheads) are found at the outermost region of the periplasm. Scale bar=0.5 μm. fg, fatty granule.



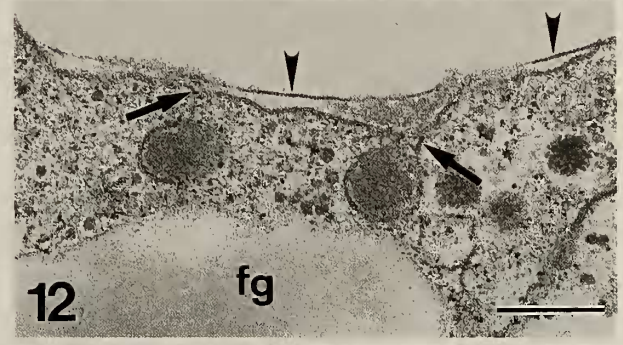


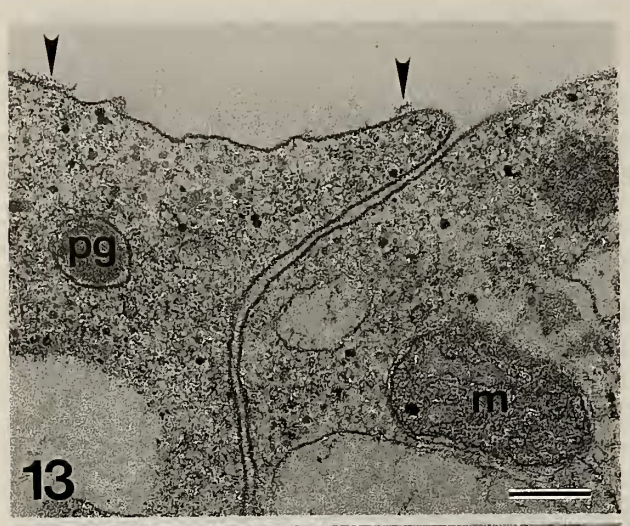
FIG. 11. The surface of the egg, three minutes after oviposition. A periplasmic granule is discharging its membrane-like contents (arrow). Discontinuous regions of mucous material are visible on the surface of the egg (arrowhead). Scale bar=0.2 μm. fg, fatty granule.

FIG. 12. The surface of the egg, three minutes after oviposition. Two periplasmic granules are discharging their very electron-dense matrix (arrows). Discontinuous thin membranes can be seen on the surface of the egg (arrowheads). Scale bar=0.2 μm. fg, fatty granule.

brane" is located within the chorion in spider eggs. According to Kondo [5], in lycosid spiders, only a thin outer layer of the "vitelline membrane", which cannot be recognized by light microscopy, is present 30 min after oviposition. The "vitelline membrane" is nearly complete at the 16-nucleus stage after formation of the main layer has started. Kondo suggested that the "vitelline membrane" bears some resemblance to the fertilization membrane in terms of its formation.

The origin of the outer layer of the fertilization membrane was clarified in the present investigation. In *A. japonica*, just at the time of oviposition, when the egg had already accepted the sperm nucleus, no structure corresponding to an outer layer was visible. Such a structure appeared for the first time as a discontinuous thin membrane or a discontinuous layer of mucous material on the surface of the egg. Fifteen minutes after oviposition, the surface of the egg was enveloped completely by a thin membrane and an underlying layer of mucous material. The contents discharged on the egg surface from the periplasmic granules appeared to form the outer layer of the fertilization membrane.

From the existence of an intermediate type of granule, we can conclude that the contents of the two types of periplasmic granule may have the same components. Both the membrane-like material and the very electron-dense



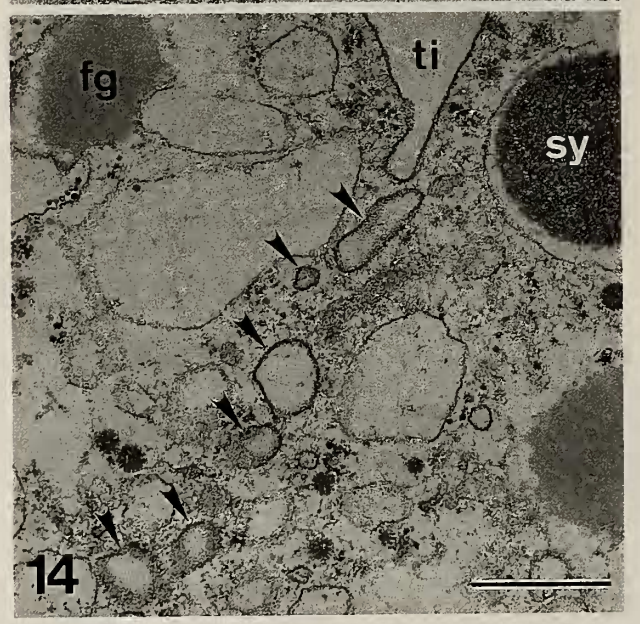


Fig. 13. Invagination of the cell membrane, three minutes after oviposition. Mucous material is visible on the surface of the egg (arrowheads). Scale bar=0.2 μm. m, mitochondrion; pg, periplasmic granule.

Fig. 14. Vesicles (arrowheads) arrayed at the tip of an invagination (ti), three minutes after oviposition. The elongation of the invagination of the cell membrane may be the result of the fusion of these vesicles. Scale bar=0.5 μm. fg, fatty granule; sy, small yolk granule.

matrix may, therefore, be able to form the mucous material. This mucous material may gradually harden from the outer side to form the outer layer of the fertilization membrane. The outer layer was observed as a membrane of 30 nm in thickness 20 min after oviposition. According to Suzuki and Kondo [13], the main layer of the "vitelline membrane" is formed by the matrix of vesicles that is discharged by exocytosis 30 min after oviposition. The formation of the outer layer and that of the main layer may be successive processes that require different materials.

In many animals other than spiders, the vitelline membrane is already complete by the time oviposition occurs.

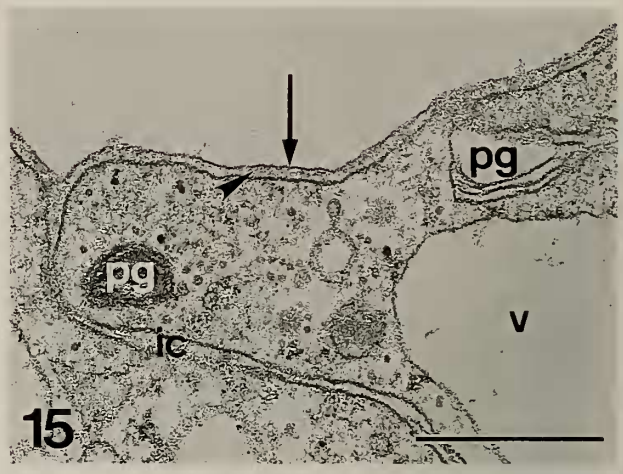


Fig. 15. The surface of the egg, fifteen minutes after oviposition. The surface is completely enveloped by a thin membrane (arrow) and an underlying layer of mucous material (arrowhead). The periplasmic granule (pg) on the right is discharging its contents. Scale bar=0.5 μm. ic, invaginating cell membrane; v, vesicle.

The formation of the outer layer after oviposition or after the entrance of the sperm nucleus suggests that the "vitelline membrane" of the spider egg is equivalent to the fertilization membrane and is not a true vitelline membrane. Suzuki and Kondo [13] postulated the incorporation of sperm into spider eggs in the ovarian cavity.

Invagination of the cell membrane and the arrangement of large yolk granules

The many microvilli found on the egg surface just at the time of oviposition may have participated in uptake of yolk materials during oogenesis. It has been reported in other spiders that the microvilli are formed at the vitellogenic stage [10–12]. In *A. japonica*, the microvilli disappeared prior to the exocytosis of periplasmic granules that resulted in formation of the outer layer of the fertilization membrane and then they appeared again prior to the formation of the main layer of the fertilization membrane.

In *A. japonica*, the cell membrane began to invaginate between 2 and 3 min after oviposition. The cell membrane that was extruded as microvilli might be utilized initially for the formation of invaginations. By contrast, the elongation of the invaginations of the cell membrane may be the result of the fusion of small vesicles, which were seen to be arrayed beyond the tip of each invagination. Some authors have reported polygonal mesh-work structures that corresponded in size to the underlying yolk masses in the periplasm in eggs of other spiders [2–4, 7, 8]. The edges of such polygons should correspond to the regions of invaginations of the cell membrane. Locy [8] and Kautzsch [3] reported that the polygons were smaller in one hemisphere than in the other in two species of *Agelena*. In *Achaearanea japonica*, no remarkable differences in the sizes of polygons were observed.

The lumen of each invagination of the cell membrane became wider and the large yolk granules were organized into

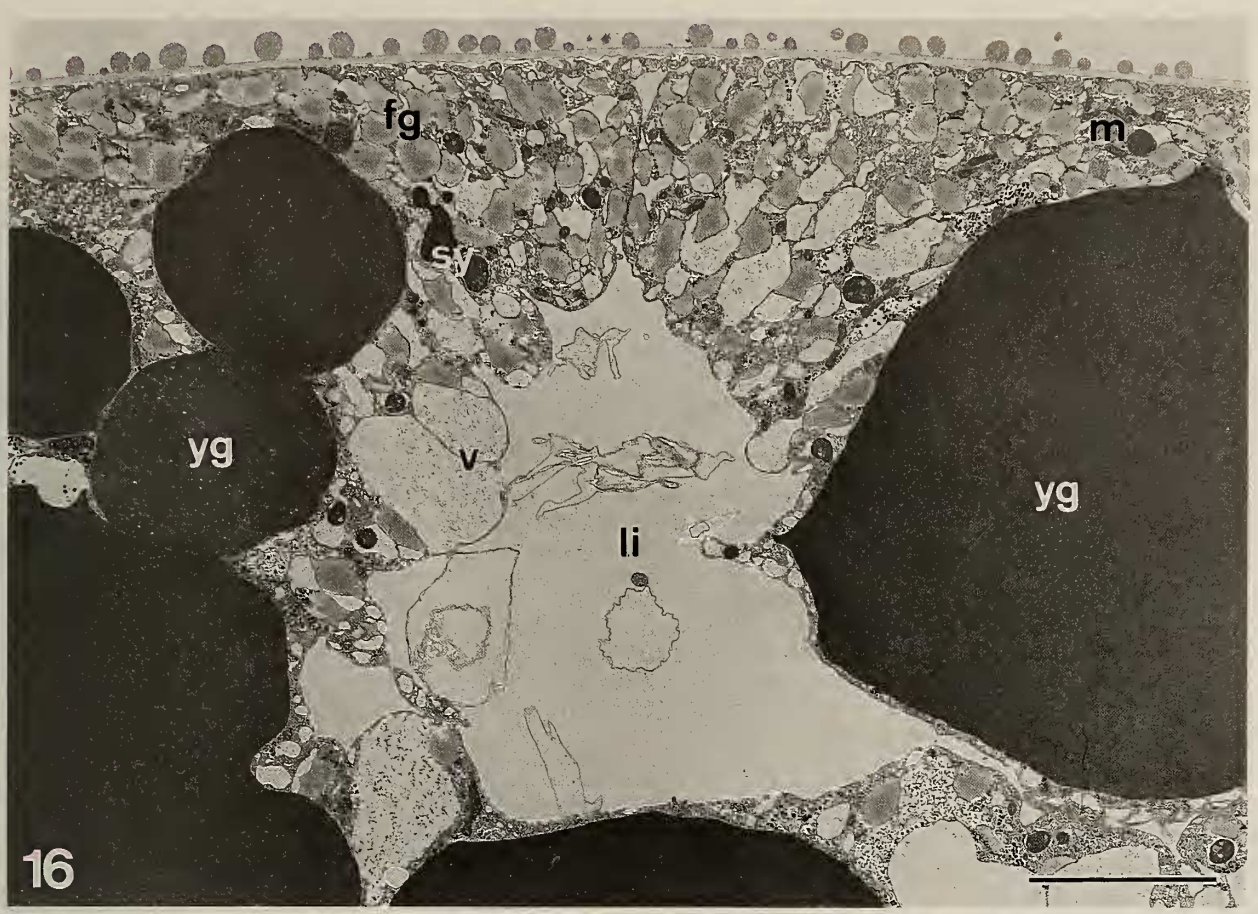


FIG. 16. Low-magnification view of the surface of an egg, twenty minutes after oviposition. The lumen of an invagination of the cell membrane (li) becomes wider and the large yolk granules (yg) become organized into radial columns in the outer region of the yolk mass (compare with Fig. 5). Scale bar=5 μm. fg, fatty granules; sy, small yolk granules; m, mitochondria; v, vesicles.

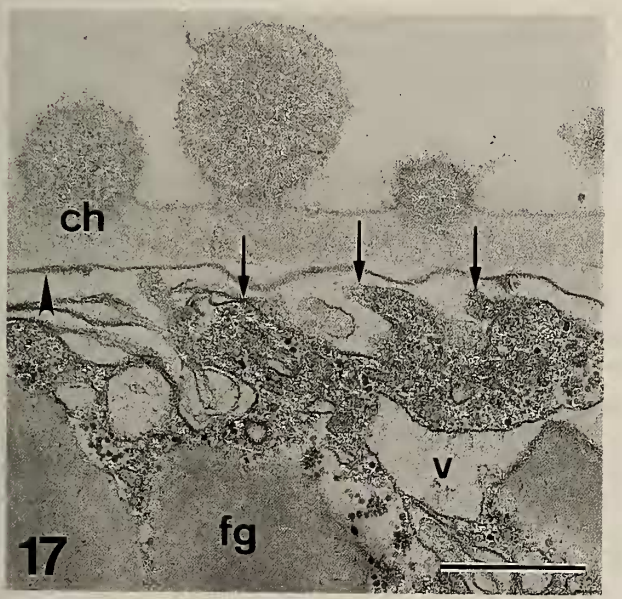


FIG. 17. High-magnification view of a portion of Figure 16. Arrowhead indicates the outer layer of the fertilization membrane. The cell membrane extrudes microvilli (arrows). Scale bar=0.5 μm. ch, chorion; fg, fatty granule; v, vesicle.

outer radial columns and an inner spherical mass. Montgomery [9] described this double structure of large yolk granules in *A. tepidariorum*. However, he failed to observe the invagination of cell membranes.

The first maturation division

Few investigations of egg maturation in spiders have been reported. Montgomery [9] described the first meiotic spindle in *A. tepidariorum*, which is oriented perpendicularly to the egg surface at the time that oviposition occurs. In *A. japonica*, the first meiotic spindle was oriented parallel to the egg surface at oviposition, and then the spindle was oriented perpendicularly to the egg surface for completion of nuclear division. This process may be explained in terms of the rotation of the meiotic spindle. The rotation may terminate prior to oviposition in *A. tepidariorum*. Fernández *et al.* [1] suggested the active participation of microtubules in the rotation of the first meiotic spindle in the leech *Theromyzon rude*.

It is unclear from the present study whether or not the first polar body is eliminated from the egg via the fusion of invaginating cell membranes. Reliable staining to the thick sections is required for further observations at the fine-

structural level.

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